APPENDIX C

Adverse Outcome Pathway as an Underlying Framework for Developing In Vitro DNT Testing Strategies

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The existing AOPs for DNT outcomes should facilitate the application of mechanistic knowledge of toxicity pathways (i.e., physiological signaling pathways perturbed upon chemical exposure) into regulatory decisions since adverse outcomes (AOs) are of regulatory relevance. Intermediate key events (KEs) represent pathways of toxicity at different biological levels (cellular, tissue and organ) which are empirically observable and measurable (Ankley et al., 2010). Therefore, in vitro DNT assays can utilize the KEs identified in DNT AOPs to detect potential developmental neurotoxicants. A battery of such in vitro test methods that relies on mechanistic KEs derived from AOPs should increase scientific confidence in *in vitro* data, and decrease uncertainty in the regulatory acceptance of in vitro assays, supporting paradigm shift towards a mechanistically-driven hazard identification and characterization and possibly risk assessment.

Furthermore, empirical evidence for describing key events relationship (KER) is based on relevant data found either from extant literature, or studies specifically designed for the purpose of AOP development. Therefore, if KERs are supported by a strong weight of evidence, in vitro assays anchored to these KEs increase confidence in the relevance of the KE to the AO. This is an important component of an IATA used in a flexible combination (fit-for-purpose) for various regulatory purposes.

Understanding the likelihood of effects (e.g., initiation of a toxicity pathway) occurring at lower, cellular levels of biological complexity through, for example in vitro testing or (Q)SAR, can also help to inform whether testing at higher levels of biological organisation (i.e., in vivo) is warranted.

Additionally, in vitro assays that allow an evaluation of the key neurodevelopmental processes specific for brain development such as cell proliferation, migration, differentiation, synaptogenesis neuronal network formation and function etc., often overlap with KEs identified in DNT AOPs, strengthening the biological context for the applied in vitro assays. Indeed, it is strongly documented in the existing literature that if these key neurodevelopmental processes are sufficiently perturbed upon exposure to a chemical, this can lead consequentially to DNT effects.

However, an approach based on individual AOPs (assays anchored to KEs) presents the limitation of being able to identify only a small number of positive “hits” (developmental neurotoxicants) eliciting toxicity through the specific AOP(s). Therefore, it has been proposed to identify key events common to many pathways described in individual AOPs. Following this recommendation, i.e., building network(s) of the existing individual AOPs relevant to DNT and determining the common KEs within such network (s), will facilitate the selection of the most critical/robust in vitro assays suitable to identify a number of developmental neurotoxicants, targeting various signalling pathways and triggered by diverse MIEs. Table

C.1 lists the assay names in the DNT in vitro battery (see Appendix B for more detailed information). Table C.2 lists the neurodevelopmental processes and other endpoints that are assessed in the DNT IVB assays. Table C.3 illustrates how the neurodevelopmental processes measured in the DNT IVB assays map to Key Events in existing developmental neurotoxicity AOPs.

Furthermore, incorporation of supplementary information delivered from DNT in vitro mechanistic studies and other alternative approaches (e.g., QSAR, read across) would increase weight of evidence when, if necessary, combined with DNT in vivo testing where results may often be equivocal or open to different interpretations with respect to whether or not a chemical has the capacity to cause DNT effects and, if so, by what mechanisms. This can be achieved by using a battery of in vitro assays which permit evaluation of a range of key pathways that mediate DNT effects, perturbed neurodevelopmental processes at different developmental exposure windows and KEs identified in the existing AOPs relevant to DNT, using human models derived from human induced pluripotent stem cells (hiPSCs), rather than rodent test systems to avoid interspecies differences.

In addition, it is important to be able to define threshold(s) for KEs (quantitative AOPs), allowing discriminating between changes observed in in vitro studies as adaptive processes normally found in biological systems in vivo, from those that are predictive of adverse outcomes. Coupling the adverse or adaptive nature of the measured endpoints with absorption, distribution, metabolism, and excretion (ADME) data and exposure information derived from in vitro to in vivo extrapolation (IVIVE) will increase the level of confidence in the information derived from in vitro assays anchored to KEs, especially if based on human neuronal/glial cells derived from hiPSCs (mimicking human biology) and coupled with models of chemical kinetics and dynamics, being more predictive for an in vivo exposure scenario.

References

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Table C.1. Assay names and neurodevelopmental processes assessed, in the DNT in vitro battery. Assay names are from Table 2.3 in the Draft Guidance Document. Note the ToxTemplate files in Appendix B contain detailed information for all the assays).

Assay Name	Process	Appendix Number
NPC1	Proliferation	Appendix B.1
NPC2a	Migration	Appendix B.2
NPC2b	Migration	Appendix B.2
NPC2c	Migration	Appendix B.2
NPC3	Neuronal Differentiation	Appendix B.2
NPC4	Neurite Outgrowth	Appendix B.2
NPC5	Glial Differentiation	Appendix B.2
UKN2	Migration	Appendix B.3
UKN4	Neurite Outgrowth	Appendix B.4
UKN5	Neurite Outgrowth	Appendix B.5
Cortical Initiation	Neurite Outgrowth	Appendix B.11
Cortical Maturation	Neurite Maturation	Appendix B.9
Cortical MEA	Neural Network Formation	Appendix B.7
Cortical Synapo	Synaptogenesis	Appendix B.9
hN initiation	Neurite Outgrowth	Appendix B.10
hNP1 Apop	NPC Apoptosis	Appendix B.6
hNP1 Prolif	NPC Proliferation	Appendix B.8

Table C.2. Neurodevelopmental processes and other endpoints in the DNT IVB assays.	
1	Proliferation
2	Neuronal differentiation
3	Neurite outgrowth (neurite length, neurite area)
4	Oligodendrocyte differentiation
5	Neural Crest Cell Migration Assay
6	Neurite Outgrowth of Neural Crest Cells
7	Neuronal network formation and function
8	Synaptogenesis
9	Cell viability
10	Cytotoxicity

Table C.3. Processes measured in the DNT IVB mapped to Key Events in developmental neurotoxicity AOPs.

AOPs *(AOP-Wiki)*	**Key Events (KEs) and corresponding in vitro assay**									
AOP 12 ***(AOP-Wiki, TFHA/WNT Endorsed)***	***Title: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development to neurodegeneration with impairment in learning and memory in aging*** *(AOP-Wiki: https://aopwiki.org/aops/12)*									
KEs	**Binding of antagonist to the NMDA Receptor (MIE)**	Inhibition of NMDA Receptor	Decreased calcium influx	Reduced levels of BDNF	Cell injury/ death	Neuro-inflammation	Neurodege-neration	**Impairment of lerning and memory (AO)**		
In vitro assays					Viability, cytotoxicity		Viability, cytotoxicity			
AOP 13 ***(AOP-Wiki, TFHA/WNT Endorsed)***	***Title: Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory ability*** *(AOP-Wiki: https://aopwiki.org/aops/13)*									
KEs	**Binding of antagonist, NMDA receptors (MIE)**	Inhibition of NMDA Receptor	Decreased calcium influx	Reduced levels of BDNF	Reduced Presynaptic Release of glutamate	Aberrant, Dendritic morphology	Cell injury & Death	Decreased Synaptoge nesis	Decreased Neuronal network function	**Impairment of lerning and memory (AO)**

In vitro assays						Neurite length and neurite area	Viabilty, Cytotoxicity	Synaptoge nesis	Neuronal network formation and function	
AOP **(under development, EFSA Scientific Opinion 2021)**	***Title: Binding of deltamethrin to Voltage Gated Sodium Channels (VGSCs) leads to the disruption action potential resulting in impairment of behavioral function (sensory motor reflex and learning)***									
KEs	**Binding to VGSC (MIE 1)** **Binding to Ryanodine receptors (MIE 2)**	Disruption of sodium channel gate kinetics hannel gate kinetics (KE1 For MIE1)	Disruption of intracellular Ca channel kinetics (KE1 for MIE2)	Disruption of action potential generation (KE 2)	Disruption of axon terminal depolarization changes in neurotrans-mitter release (KE3)	Decreased oligodendro-cytes differentiation (KE5)	Increase of intracellular sodium in microglia cells. (KE6)	Altered neuronal network function (KE4)	**Impairment behavioural function (AO)**	
In vitro assays				Neuronal network formation and function	Neuronal network formation and function	Oligodedro-cytes differentiation		Neuronal network formation and function		
AOP 42 ***(AOP-Wiki, TFHA/WNT Endorsed)***	***Title: Inhibition of Thyroperoxidase and Subsequent Adverse Neurodevelopmental Outcomes in Mammals*** *(AOP-Wiki: https://aopwiki.org/aops/42)*									
KEs	**Thyroperoxidase Inhibition (MIE)**	TH synthesis, Decreased	T4 in serum, Decreased	T4 in neuronal tissue, Decreased	Hippocampal gene expression, Altered	Hippocampal anatomy, Altered	Hippocampal Physiology, Altered	**Cognitive Function, Decreased (AO)**		
In Vitro assays							Synapto-genesis, Neuronal network			

							formation and function			
AOP *(outlined in Hassan et al., 2017)*	***Thyroid hormone (TH) synthesis inhibition and development of a cortical brain malformation, a cortical heterotopia***									
KEs	**Thyroperoxidase inhibition (in dam and Fetus) (MIE)**	TH release in dam and fetus decreased	TH in serum of dam and fetus decreased	TH in fetus brain decreased	**Cortical heterotopia (AO)**					
In vitro assays										
AOP 54 *(AOP-Wiki, TFHA/WNT Endorsed)*	***Title: Inhibition of Na+/I− symporter (NIS) decreases TH synthesis leading to learning and memory deficits in children*** *(AOP-Wiki: https://aopwiki.org/aops/54)*									
KEs	**Inhibition, Na+/I-symporter (NIS) (MIE)**	Thyroidal Iodide, Decreased	TH synthesis, Decreased	T4 in serum, Decreased	T4 in neuronal tissue, Decreased	BDNF, Reduced	GABAergic interneurons, Decreased	Synapto-genesis, Decreased	Neuronal network function, Decreased	**Impairment, Learning and memory (AO)**
In vitro assays								Synapto-genesis	Neuronal network formation and function	
AOP 8 *(AOP-Wiki, under development)*	***Upregulation of Thyroid Hormone Catabolism via Activation of Hepatic Nuclear Receptors, and Subsequent Adverse Neurodevelopmental Outcomes in Mammals***									
KEs	**Xenobiotic nuclear receptor activation (MIE)**	Increased Phase II catabolism/ Increased hepatic transport	Decreased T4/T3 serum levels	Decreased tissue TH concentration	Altered neuro-development	**Neurological dysfunction (AO)**				

In vitro assays										
AOP 17 ***(AOP-Wiki, EAGMST Under Review)***	***Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory***									
KEs	**Binding to Thiol/seleno-proteins involved in protection against oxidative stress (MIE)**	Decreased protection against oxidative stress	GSH depletion	Oxidative stress	Glutamate dyshomeo-stasis	Cell injury/death	Neuroinfla-mmation	Tissue resident cell activation/ Increased Pro-inflamma-tory mediators	Decrease of neuronal network function	**Impairment, learning and memory (AO)**
In vitro assays						Viability Cytotoxicity			Neuronal network formation and function	
AOP 134 ***(AOP-Wiki, under development)***	***Sodium Iodide Symporter (NIS) Inhibition and Subsequent Adverse Neurodevelopmental Outcomes in Mammals***									
KEs	**Inhibition, Na+/I-symporter (NIS) (MIE)**	Thyroid hormone synthesis, Decreased	Decrease of Thyroidal iodide	Thyroxine (T4) in serum, Decreased	Thyroxine (T4) in neuronal tissue, Decreased	Hippocampal gene expression, Altered	Hippocampal anatomy, Altered	Hippocam pal Physiology , Altered	**Cognitive Function, Decreased (AO)**	
In vitro assays								Neuronal network formation and function		

AOP 152 *(AOP-Wiki, under development)*	***Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity***									
KEs	**Binding, Transthyretin in serum (MIE)**	Serum thyroxine (T4) from transthyretin Desplace-ment	Increased, Free serum thyroxine (T4)	Increased, Uptake of thyroxine into tissue	Increased, Clearance of thyroxine from serum	T4 in serum, Decreased	T4 in neuronal tissue, Decreased	Hippocam pal gene expression , Altered	Hippocampa l anatomy and pyysiology Altered	**Cognitive Function, Decreased (AO)**
In vitro assays										
AOP 275 (AOP-Wiki, under development)	***Histone deacetylase inhibition leads to neural tube defects***									
KEs	**Histone deacetylase inhibition (MIE)**	Histone acetylation, increase	Altered, Gene Expression	Altered differentiat ion	**Neural tube defects (AO)**					
In vitro assays				Neurite length and neurite area						
AOP 300 (AOP-Wiki, under development)	***Thyroid Receptor (TR) Antagonism and Subsequent Adverse Neurodevelopmental Outcomes in Mammals***									
KEs	**TR Antagonism (MIE)**	Hippocampal gene expression, Altered	Hippocampa l anatomy, Altered	Hippocam pal Physiology , Altered	**Cognitive Function, Decreased (AO)**					

In vitro assays										
AOP *(US EPA report, 2017)*	***Inhibition of deiodinase results in decreased thyroxine (T4) to triiodothyronine (T3) conversion and subsequent adverse neurodevelopmental outcomes***									
KEs	**Deiodinase inhibition (MIE)**	Decreased T4 and/or T3 in target tissues	TR binding/trans-activation	Gene expression modifications	Neurodevelop-mental Alterations	**Neurological and cognitive impairments (AO)**				
In vitro assays										
AOP *(US EPA report, 2017)*	***Interference with thyroid receptor and subsequent adverse neurodevelopmental outcomes***									
KEs	**Thyroid receptor binding (MIE)**	Decreased T4 and/or T3 in target tissues	TR binding/trans-activation	Gene expression modifications	Neurodevelop-mental Alterations	**Neurological and cognitive impairments (AO)**				
In vitro assays										
AOP *(US EPA report, 2017)*	***Interference with thyroid hormone transport results in decreased T4 in brain and subsequent adverse neurodevelopmental outcomes***									
KEs	**Thyroid hormone transport interference (MIE)**	Decreased T4 and/or T3 in target tissues	TR binding/trans-activation	Gene expression Modifica-tions	Neurodevelo-pmental Alterations	**Neurological and cognitive impairments (AO)**				

In vitro assays										
AOP III *(Cristina Suñol In Bal-Price et al., 2017)*	***Binding of antagonist to GABA A receptor results in hyperexcitability and convulsions***									
KEs	**Binding of antagonists to GABAA Receptor (MIE)**	Inhibition of $GABA_A$ Receptor	Reduced/ blocked CL^- influx	Reduced inhibitory signals	Disinhibition of Excitatory pathways	Increased excitatory activity in neuronal network	**Seizures/ Convulsions (AO)**			
In vitro assays						Neuronal network formation and function				
AOP IX *(Pamela J. Lein in Bal-Price et al., 2017)*	***The interaction of non-dioxin-like PCBs with ryanodine receptors (RyRs) causes their sensitization affecting neuronal connectivity that results in behavioral deficitsiBinding to RyRcits***									
KEs	**Biding to RyRs (MIE)**	RyRs sensitization	Altered neuronal calcium oscillations	Activation calcium dependent signalling	Altered dendritic arborization and synapto-genesis	Increased neuronal apoptosis	Altered neuronal networks and pathways	**Behavioral deficits (learning, memory, psychomotor, attention) (AO)**		
In vitro assays					Neurite length and neurite area, Synapto-genesis	Viability, cytotoxicity	Neuronal network formation and function			
AOP *(outlined in von Stackelberget al., 2015)*	***Exposure to Mixtures of Metals and Neurodevelopmental Outcomes***									

KEs	**Activationof MEK-ERK1/2 in Astrocytes (MIE)**	Calcium Signaling	ROS Generation and Oxidative Stress	Apoptosis in Astrocytes	Neurotrans-mitter Release	**Learning and Cognitive Deficits (AO)**				
In vitro assays				**Viability, cytotoxiciy**						
AOP *(outlined in von Stackelberget al., 2015)*	***Exposure to Mixtures of Metals and Neurodevelopmental Outcomes***									
KEs	**Multiple potential events for individual metals (MIEs)**	Mitochon-drial dysfunction	Reactive oxygen species/oxid ative stress	Apptosis	Synapse impairment; neurodegene-ration	**Learning and Cognitive Deficits (AO)**				
In vitro assays				Viability, cyto-toxicity	Synapto-genesis/ Viability, cytotoxicity					
AOP *(outined in Barenys et al., 2020)*	***Interference of a compound with the serotonin transporter and enzyme synthesis during brain development leading to decreased motor functions in children***									
KEs	**Unknown SERT interaction and TPH inhibition? (MIE)**	Depletion of 5HT	Impaired trophic 5HT function during neurodeve-lopment	KE3 KE 3.1, KE 3.2, KE 3.n unknown	Altered neuroanatomi cal structure	**Decreased motor function in children (AO)**				
In vitro assays										

AOP *(outined in Barenys et al., 2020)*	***Alteration of DA receptor signaling during development leading to a cortical imbalance of excitatory and inhibitory neurons in cortex causing decreased motor functions in children***									
KEs	**Unknown (MIE)**	Alteration of dopamine receptors signaling during development	Altered tangential migration of GABAergic neurons	Cortical imbalance of excitatory and inhinbitory neurons	Unknown	**Decreased motor function in children (AO)**				
In vitro assays										